Synopsis

Supporting Cell Mediated Homeostasis of Chick Basilar Papilla for Hair Cell Restoration

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Introduction

Auditory dysfunction can result from the loss of sensory hair cells (HCs) in the organ of Corti due to chronic noise exposure, ototoxic substances, and aging (Coleman, 1976; McGill and Schuknecht, 1976). Non-mammalian vertebrates, such as birds, have HC regenerative abilities, a property that has been lost in mammals (Corwin and Cotanche, 1988; Cotanche, 1987; Cruz et al., 1987; Ryals and Rubel, 1988). In birds, HC regenerate from a second cell type found in the auditory epithelium, known as supporting cells (SC). In normal physiology, SC serve diverse functions, such as mechanical and metabolic support, as well as maintaining ionic homeostasis during physiological processes (Monzack and Cunningham, 2013). In birds, SC can regenerate HC through one of two mechanisms: direct trans-differentiation or, more rarely, through asymmetric mitotic division (Shang et al., 2010; Stone and Cotanche, 2007). These events are initiated when HC are lost, however the steps involved in SC activation, and a clear dissection of the pathways that are involved in HC regeneration, have remained elusive. Parallels with HC differentiation suggest the role of various signaling pathways. Notably, the interaction between the Wnt and Notch signaling pathways, extensively studied in cochlear development and regeneration, plays a crucial role (Jacques et al., 2014; Zak et al., 2015).

In chick, hair cells become apparent from embryonic day (E)6, starting from a small circular patch at the distal end of the basilar papilla (BP) and extending proximally (Goodyear and Richardson, 1997). The number of differentiated HCs increases until E10, after which HC number stabilize and show a moderate increase until E12 (Goodyear and Richardson, 1997). HC themselves arise from a prosensory domain, a group of sensory precursors that express the transcription factor Sox2 which is necessary for HC formation (Kiernan et al., 2005b; Neves et al., 2007; Pan et al., 2013). Both Notch and Wnt signaling can impact the regulation of Sox2:

Notch, through its interaction with its ligand Jag1, positively regulates Sox2 expression (Brooker et al., 2006; Daudet et al., 2007; Kiernan et al., 2006). The Wnt pathway, through its downstream effector ß-catenin, has been shown to upregulate Sox2 expression and promote proliferation (Jacques et al., 2014; Jacques et al., 2012), however more recent data suggest that this regulation depends on the exact levels of Wnt activity, with low levels of signalling necessary for Sox2 and high levels being inhibitory (Zak and Daudet, 2021). Wnt signalling is also thought to activate the transcription factor, Atoh1, in the prosensory region. Atoh1 is necessary and sufficient for hair cell differentiation, and initially, all cells in the prosensory region express Atoh1 (Bermingham et al., 1999; Driver et al., 2013; Stone et al., 2003). However, HC differentiation is limited to specific cells through the action of Notch, this time with its ligands Dll1 and Jag2 (Kiernan et al., 2005a; Lanford et al., 1999). This interaction releases the Notch intracellular domain (NICD), which translocates to the nucleus to activate target gene transcription. In this case, NICD activates a repressor of HC differentiation, specifying these cells as Supporting Cells (SC) and allowing neighbouring cells to develop as HC, creating a mosaic of Notch-ligand expressing HC to be surrounded by Notch-active SC.

While differentiation is occurring, the BP is undergoing considerable cellular rearrangements as convergence and extension movements extend the epithelium and drive the allignment of intrinsic polarity (Goodyear and Richardson, 1997; Prakash et al., 2023). On average SC lose SC neighbours as they reorganize around HC. Cell rearrangements and the increase in the apical surface area of hair cells have been proposed to account for much of these changes. However, there remains the possibility that some plasticity in cell identity exists that allows the HC-SC mosaic to be maintained in the BP, even while cellular rearrangement occurs. To understand this, we used an electroporation approach to knock-down Atoh1 mosaically, in the otocyst of the chick. We find that at E9, electroporation leads to the loss of HC. However, by E12 HC number is restored. Using inhibitor studies and by introducing reporter constructs, we find that both Wnt and Notch pathways are involved in HC restoration. We suggest that there is a second wave of HC differentiation, that occurs during BP cellular rearrangements that ensure cellular pattern is maintained.

Materials and Methods

The comprehensive description of the materials and methodologies utilized in this research can be found in the publication; Singh, N., Prakash, A., Chakravarthy, S.R., Kaushik, R., Ladher, R.K., 2022. ‘In Ovo and Ex Ovo Methods to Study Avian Inner Ear Development. Journal of

visualized experiments' and in Singh, N., Kaushik, R., Prakash, A., Saini, S., Garg, S., Adhikary, A., Ladher, R.K., 2024. 'Mosaic Atoh1 Deletion in the Chick Auditory Epithelium Reveals a Second Period of Differentiation to Restore Hair Cell Number. http://dx.doi.org/10.2139/ssrn.4814834'.

Results

Hair Cells are lost in Atoh1-crispant Basilar Papilla

The right otic vesicle of an E3.5-4 embryo was electroporated with either a control-gRNA or Atoh1-gRNA, (Fig. 1A & 1B) with the left otic vesicle as the internal control. Embryos were incubated until E9-E12, at which point the BP was dissected and HC development assessed.

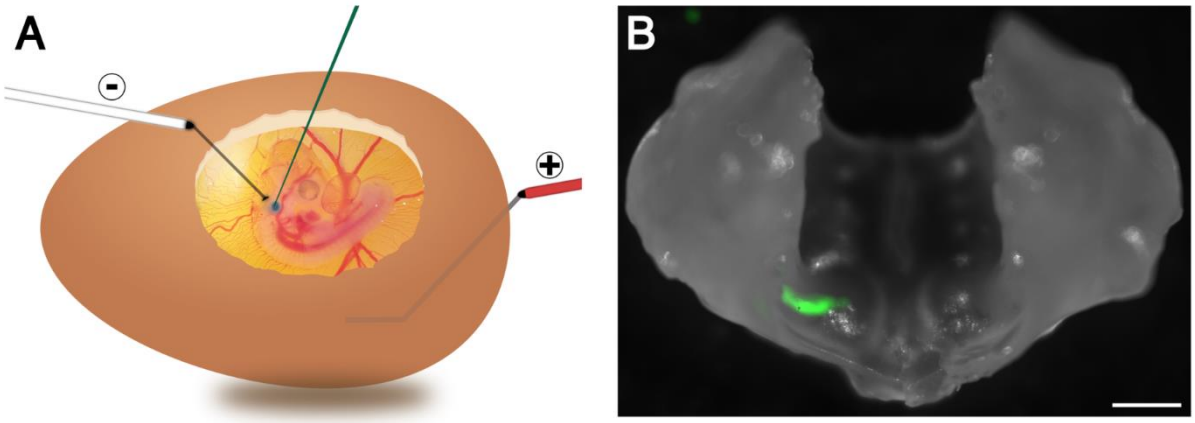


Figure 1: In Ovo Electroporation of guide RNA and Tol2-eGFP plasmids at E4. (A) Schematic illustrating the technique of in ovo microinjection and electroporation into the chick otic vesicle at embryonic day 4 (E4). (B) The left inner ear serves as an internal control. The GFP expression is observed in the E10 cochlear duct of the right inner ear, scale bar is 1.5 inches.

As the spatial arrangement of HCs and SCs varies along the length of BP from proximal to distal end as well as during development, we independently assessed the three regions (Figure 2A) for each embryonic stage (Goodyear and Richardson, 1997; Prakash et al., 2023). At E9 stage, the Atoh1-crispant BP shows a reduced number of HCs when compared to both control and control-gRNA BP (Fig. 2B-D). However, we find that, by E12, no difference in HC number was observed (Fig. 2E-G). The presence of the tracer GFP allowed us to assess the extent of electroporation. We noted that at E9, in the Atoh1-crispants, patches of missing HCs coincided with areas where GFP expression is observed (Fig. 2D). By E12, despite the presence of GFP, HC numbers had recovered (Fig. 2H-K). However, upon close inspection, we noticed that recovered HC were not GFP positive (Fig. 2G). To quantitate this, we quantified the number of cells that co-expressed GFP and the hair cell marker, Myosin 7a. We found that

when compared to control-gRNA samples, significantly fewer Myo7a/GFP double-positive cells in Atoh1-crispants (Fig. 2L).

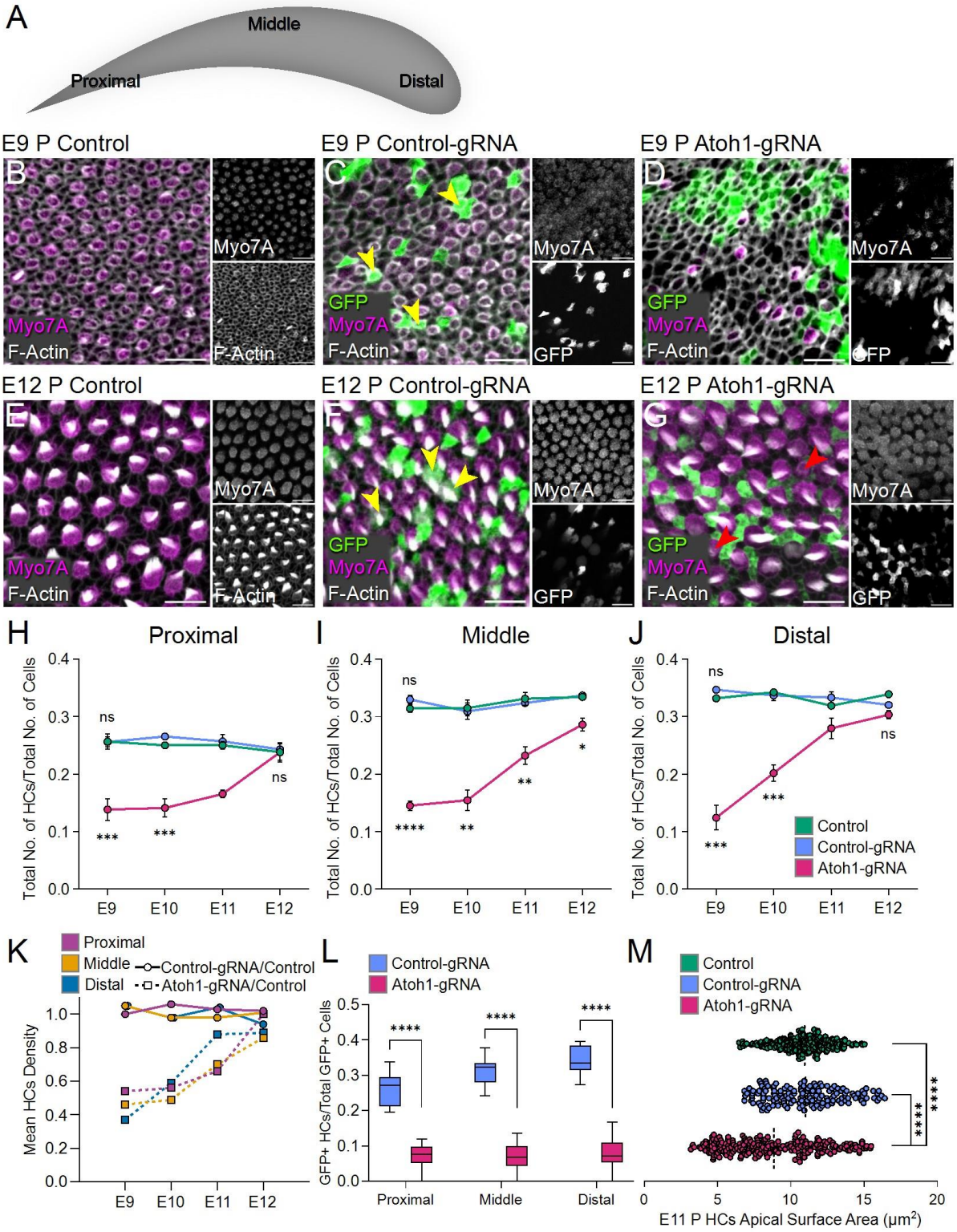


Figure 2: Mosaic Atoh1 deletion results in loss of hair cells at E9 & subsequent recovery by E12. (A) Schematic of BP divided into three regions proximal, middle and distal. Images (B-G) are obtained from the proximal region of the BP (Scale bar is 10μm). (B-D) are from chick embryo E9 and (E-G) are from chick embryo E12. (B)

*Contralateral Control E9 BP showing phalloidin-stained F-Actin (gray) and Myo7a-labeled hair cells (HCs) (magenta). (C) Control-gRNA electroporated E9 BP showing GFP electroporated cells (green), phalloidin-stained F-Actin (gray) and Myo7a-labeled hair cells (HCs) (magenta). Yellow arrowheads mark GFP-positive HCs. (D) Atoh1-gRNA electroporated E9 BP showing GFP electroporated cells (green), phalloidin-stained F-Actin (gray) and reduced Myo7a-labeled hair cells (HCs) (magenta). (E) Contralateral Control E12 BP showing phalloidin-stained F-Actin (gray) and Myo7a-labeled hair cells (HCs) (magenta). (F) Control-gRNA electroporated E12 BP showing GFP electroporated cells (green), phalloidin-stained F-Actin (gray) and Myo7a-labeled hair cells (HCs) (magenta). Yellow arrowheads mark GFP-positive HCs. (G) Atoh1-gRNA electroporated E9 BP showing GFP electroporated cells (green), phalloidin-stained F-Actin (gray) and restored Myo7a-labeled hair cells (HCs) (magenta). Red arrowheads mark new born HCs, identified based on size. (H - J) Graph showing the number of HC as a proportion of the total number of cells in contralateral control (green), Control-gRNA (blue) and Atoh1-gRNA (red), at four stages (E9, E10, E11 and E12), in the proximal (H), middle (I) and distal (J) regions of the BP. (**** P<0.0001; *** P < 0.001; ** P < 0.01 * P < 0.05; ns = non-significant). (K) Graph showing the density of HC, compared to that of the contralateral control, of Control-gRNA (solid line) and Atoh1-gRNA (dashed line) in proximal (magenta), middle (orange) and distal (blue) at E9-12. (L) The graph showing ratio of GFP-positive HCs to the total number of GFP-positive cells in different regions of the BP for Control-gRNA (blue) and Atoh1-gRNA (red). (M) The graph shows apical surface area of HCs at proximal region of E11 BP (**** P<0.0001)*

To understand the time course of hair cell restoration, we examined the BP of embryos at E9, 10, 11 and 12. We found that the rate of hair cell restoration appears distally first followed by more proximal regions, mirroring the natural process of hair cell differentiation in the BP (Fig. 2K). At E12, Atoh1-crispants show restored hair cell and support cell arrangement resembling that of both the control and control-gRNA groups. In Atoh1-crispants, GFP is predominantly confined to supporting cells. (Fig. 2G). Moreover, the apical surface area of the restored hair cells is smaller compared to those in the control basilar papilla (Fig. 2M), with some lacking hair bundles but, nonetheless, expressing Myo7a.

Neighbourhood Analysis

HC and SC are mosaically organized in the BP. HC contact SC (HC-SC contact), but very rarely another HC (HC-HC contact) (Goodyear and Richardson, 1997; Prakash et al., 2023). SC can contact HC (SC-HC contact) and SC (SC-SC contact) (Fig. 3A). To understand whether HC restoration is accompanied by a restoration in the mosaic pattern of the BP, we conducted neighbour number analysis for both HC and SC between E9 and E12.

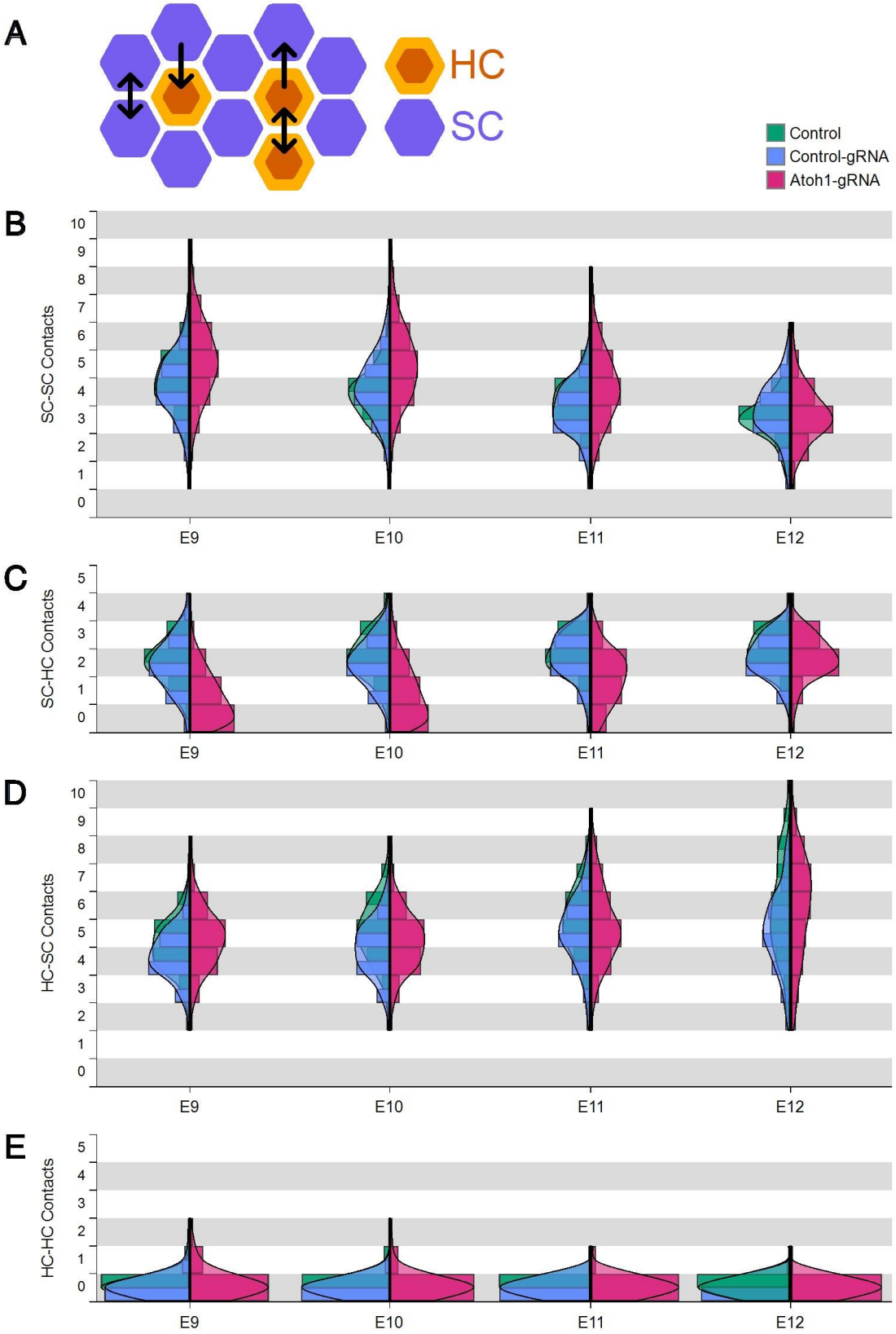


Figure 3: Neighborhood connections: HC and SC interaction analysis. (A) The type of interactions between HCs and SCs. The arrows symbolise four types of connections between these two cell types: SC-SC, SC-HC, HC-SC,

and HC-HC. (B-E) The split-histograms show the number of neighboring cells for each cell type in contralateral control (green), control-gRNA (blue), and Atoh1-gRNA (red) at four embryonic stages from E9 to E12. (B) Number of SC-SC contacts, (C) Number of HC contacted by a single SC, (D) Number of SC contacted by a single HC, (E) Number of contacts made between HC-HC.

As expected, at E9 Atoh1-gRNA electroporated BP show a greater number of SC-SC contacts, with dramatically reduced SC-HC contacts given the reduced HC numbers in electroporated patches. While around 3-5% of SC are without an HC contact in control and control-gRNA samples, 47% of SC do not have an HC contact in Atoh1-gRNA samples (Fig. 3C). The reduction in SC-SC contacts and the recovery of SC-HC contacts occurs by E12 such that, the number of both SC-SC and SC-HC contacts are similar to those seen in control-gRNA samples (Fig. 3B & 3C). Consistent with this, the number of SC without HC contacts falls to control-gRNA levels by E12.

At E9, substantial HC-HC contacts are observed in Atoh1-gRNA samples. These are rarely observed in control samples (Fig. 3E). These contacts resolve by E12. Surprisingly, the number of HC-SC contacts showed an increase at E12 (Fig. 3D). This may be due to the smaller apical surface area of restored HC.

Proliferation Status of Basilar Papilla After HC Loss

We next asked if HC restoration was associated with changes in proliferation. Embryos electroporated with either control-gRNA or Atoh1-gRNA were grown to E9, E10, E11, and E12. At these stages the BP was explanted and cultured for one day in the presence of EdU (Fig. 4A). After culture, explants were fixed and processed for Sox2 (as a support cell marker) and Myo7a (a hair cell marker) immunostaining, as well as EdU incorporation. Sections of explanted BP showed a clear demarcation between hair cells (HCs) and supporting cells (SCs). HCs were situated closer to the luminal surface, while SC nuclei were grouped closely near the basilar membrane, making them easily distinguishable and countable.

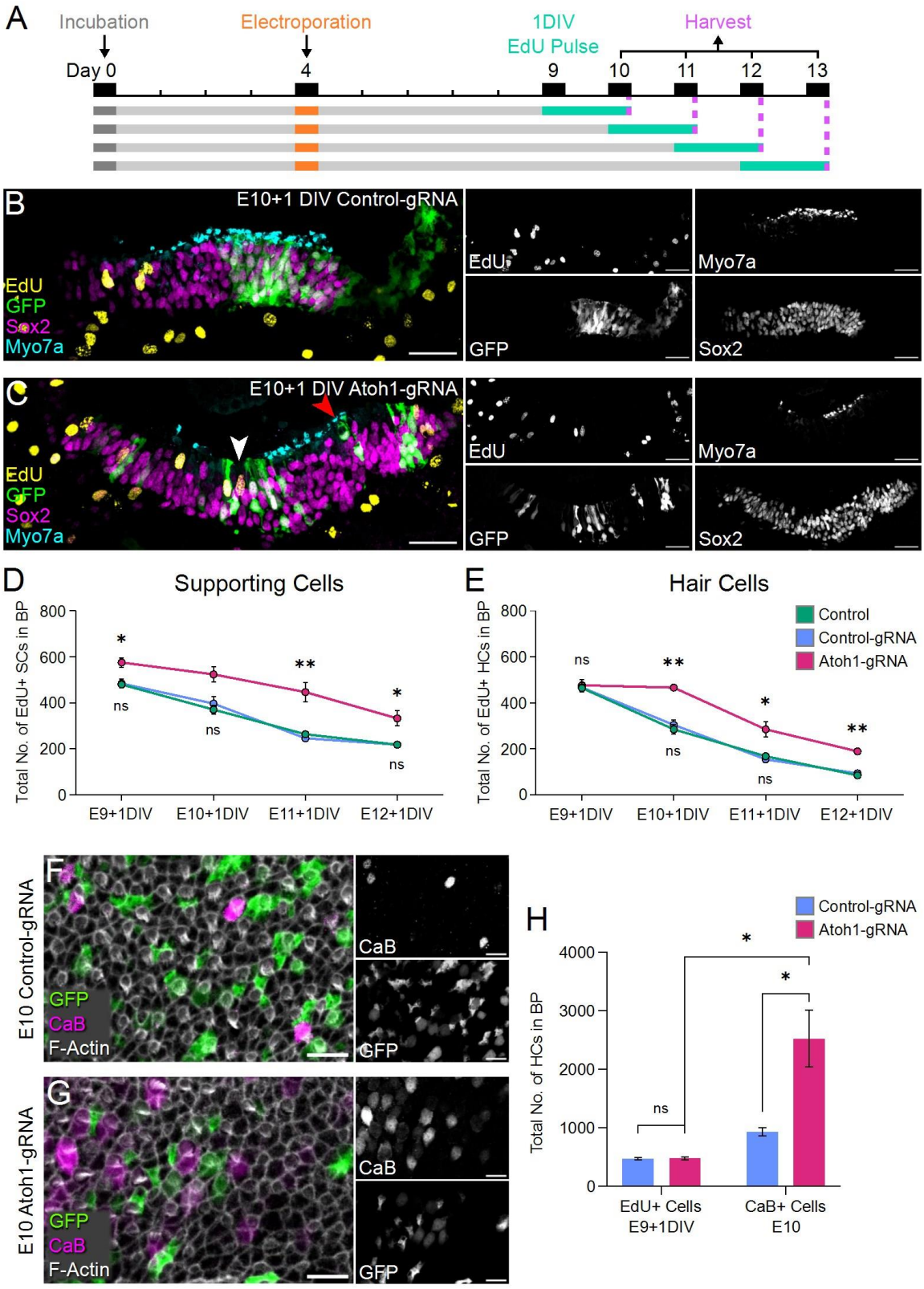


Figure 4: Proliferation dynamics in developing BP. (A) Illustration of the experimental design for EdU pulse treatment. Electroporation is performed on all embryos at E4. At the specified stages of E9, E10, E11, and E12, a one-day EdU pulse was administered through in vitro BP explants, followed by the harvesting of the BP. (B-C)

*Cross-sectional images of E10+1-day in vitro (DIV) BP explants, (Scale bar is 25µm). Myo7a staining HCs in cyan, Sox2 staining SCs in magenta, EdU labeling in yellow, and green indicating GFP expression in Control-gRNA (B) and Atoh1-gRNA BP (C). The white arrow marks the region with missing HCs with an EdU-positive SC beneath it, and the red arrow marks GFP-positive HCs, referred to as "escapers". (D) Graph showing EdU-positive SCs and (E) EdU-positive HCs from embryonic stages E9+1 DIV to E12+1 DIV from control, control-gRNA, and Atoh1-gRNA. (F-G) are whole mount images of E10 BP from electroporated chick embryos (Scale bar is 10µm). Calbindin (CaB)(magenta) marks new HCs phalloidin staining F-Actin (grey), and GFP expression (green) of control-gRNA at E10 (F) and Atoh1-gRNA BPs (G). (H) Graph showing the total number of HCs in both E9+1 DIV BP explant (treated with EdU) and E10 BP with two condition groups: control-gRNA (blue) and Atoh1-gRNA (magenta). *P<0.05 and **P<0.01, ns - non-significant.*

In BP sections, a higher number of EdU-positive cells were observed at E10+1 DIV, coinciding with GFP expression in Atoh1-crispants (Fig. 4C). In contrast, the control-gRNA exhibited sporadic presence of EdU-positive cells (Fig. 4B). At each stage from E9+1 DIV to E12+1 DIV, we detected increased numbers of EdU-positive cells in Atoh1-gRNA electroporated regions, when compared to corresponding regions in control-gRNA or unelectroporated BPs. The numbers of Sox2/EdU double positive cells are enhanced in the Atoh1-gRNA crispants, however the numbers show a gradual decline between E9+1 DIV to E12+1 DIV (Fig. 4D). Myo7a/EdU positive cells show comparable levels between Atoh1-gRNA and control BPs at E9+1 DIV, however at E10+1 DIV there is a significant elevation in these numbers when compared to controls (Fig. 4E). This suggests the generation of new hair cells between E9 and E10 in response to Atoh1-gRNA electroporation. In agreement with this, we could detect Myosin7a and Sox2 double-positive cells in Atoh1-gRNA crispants, suggesting the emergence of new hair cells.

To confirm the presence of newly generated hair cells, we immunostained the BP with Calbindin (Fig. 4F & 4G), which labels newborn hair cells at E10, and compared it to the EdU positive hair cells data from E9+1 DIV Atoh1-gRNA electroporated explants (Chen et al., 2021; Cox et al., 2014). We find significantly more Calbindin-positive (CaB+) new hair cells than EdU-positive hair cells at the same developmental stage (Fig. 4H). This suggests the involvement of additional mechanisms in HC restoration, such as transdifferentiation of supporting cells. In agreement with this, we observed a substantial population of cells within Atoh1-gRNA samples that exhibited co-expression of Myosin7a and Sox2, markers typically associated with newly generated hair cells. These cells did not show EdU incorporation. These findings suggest that hair cell restoration could involve both direct transdifferentiation from supporting cells into hair cells and mitotic division of supporting cells. Transdifferentiation likely initiates earlier at E9+1DIV, while mitotic division becomes more prominent later. This finding aligns with previous research indicating that mitotic division of supporting cells could

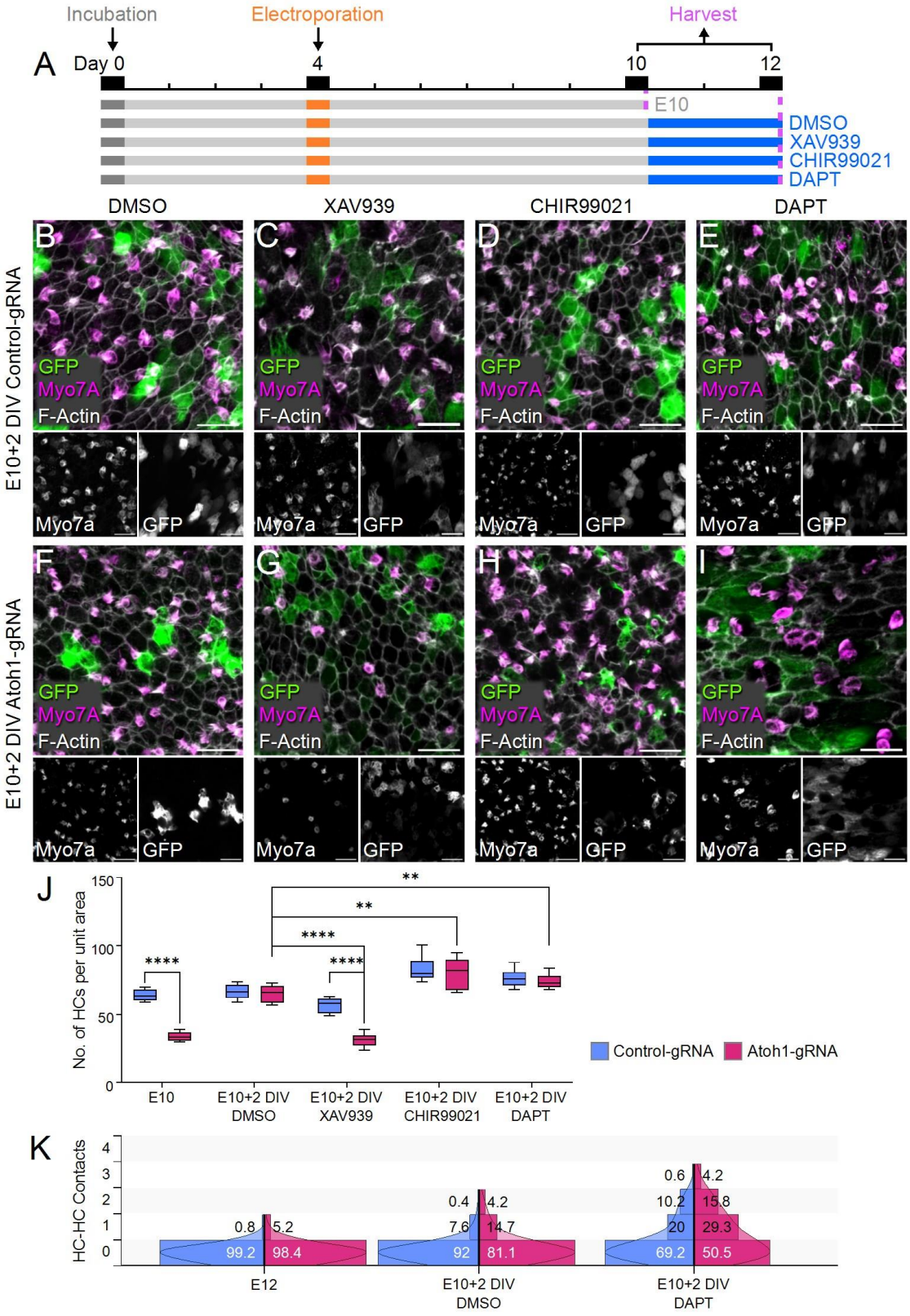
play a role in the restoration of basilar papilla tissue (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Although the primary mode of hair cell regeneration in chick basilar papilla is reported to be direct transdifferentiation of supporting cells (Shang et al., 2010; Stone and Cotanche, 2007).

Molecular Regulators of HC Restoration

To understand the molecular mechanisms that could be involved in HC restoration, we used our explant approach as a proof of principle to screen modulators of signalling pathways that might influence HC restoration. Given the correspondence, we tested small molecules that modulate pathways involved in HC regeneration.

Wnt signalling has been implicated in the development of HC (Jacques et al., 2012; Shi et al., 2014). Activators of the Wnt/ß-catenin pathway increase the proliferation of prosensory cells in the organ of Corti, in the chick BP as well as in zebrafish neuromasts. Wnt activation has also been shown to potentiate regeneration in the zebrafish lateral line, and genes associated with this pathway are robustly induced upon neuromast damage (Head et al., 2013; Jiang et al., 2014). We thus used an inhibitor and activator of canonical Wnt signalling to ask if HC restoration was affected.

Control-gRNA and Atoh1-gRNA were electroporated into the E4 otocyst with a tracer construct. At E10 the BP was dissected. It was then either immediately fixed (E10+0) or treated with XAV939, a canonical Wnt antagonist, or CHIR99021, a Wnt agonist. Explants were cultured for 2 days (Fig. 5A), then fixed and HC and SC numbers assayed.


A
Incubation
Electroporation
Harvest
Day 0
4
10
12
E10
DMSO
XAV939
CHIR99021
DAPT
DMSO
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CHIR99021
DAPT
E10+2 DIV Control-gRNA
E10+2 DIV Atoh1-gRNA
B
C
D
E
F
G
H
I
GFP
Myo7A
F-Actin
Myo7a
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No. of HCs per unit area
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100
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Control-gRNA
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E10
E10+2 DIV DMSO
E10+2 DIV XAV939
E10+2 DIV CHIR99021
E10+2 DIV DAPT
K
HC-HC Contacts
4
3
2
1
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0.8
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92
81.1
0.6
4.2
10.2
15.8
20
29.3
69.2
50.5
E12
E10+2 DIV DMSO
E10+2 DIV DAPT

*Figure 5: Wnt and Notch Pathway Modulation in Atoh1-Crispant Basilar Papilla. (A) Diagram illustrating the experimental setup for the treatment with pharmacological inhibitors and activators. Electroporation is performed on all embryos at E4. At the E10 embryonic stage, a two-day treatment with small molecules is administered through in vitro basilar papilla explants, followed by harvesting the basilar papilla at E10+2 DIV. (B-I) Images of basilar papilla explants from electroporated chick embryos showing F-Actin (gray), Myo7a-labeled hair cells (magenta), and GFP (green). Scale Bar is 10μm. The top panel (B, C, D, E) displays images from basilar papilla explants of control-gRNA electroporated otocysts. The lower panel (F, G, H, I) shows images from the basilar papilla explants of Atoh1-gRNA electroporated otocysts. Explants are treated with DMSO vehicle control (B, F); the Wnt antagonist XAV939 (C, G); the Wnt agonist CHIR99021 (D, H) and an inhibitor of Notch signalling, DAPT (D, H). (J) The graph illustrates the number of hair cells in a unit area in control-gRNA and Atoh1-gRNA basilar papilla explants. E10, serves as zero time point before treatment starts. To assess statistical significance in direct comparisons between the two conditions, Student's t-tests were conducted to calculate P-values, with **P<0.01 and ****P<0.0001 indicating statistical significance. (K) The graphs shows the number of HC-HC contacts in electroporated control-gRNA, and Atoh1-gRNA with E12 control basilar papilla and E10+2 DIV (DMSO & DAPT) treated basilar papilla explant.*

Treatment with XAV-939 blocked the restoration of HC in Atoh1-gRNA electroporated BP explants, with numbers similar to those seen at E10 (Fig. 5G & 5J). In contrast, control-gRNA explants treated with XAV-939 showed normal numbers of HC (Fig. 5C & 5J). In contrast, CHIR99021-treatment did not affect HC restoration in Atoh1-gRNA electroporated E10+2 DIV BP explants (Fig. 5H & 5J). Indeed, when compared to vehicle control (Fig. 5B & 5F), there was mild elevation of HC numbers in both control-gRNA and Atoh1-gRNA electroporated explants after CHIR99021-treatment (Fig. 5J). Taken together, these data suggest that Wnt signalling may play in an aspect of HC restoration.

We next turned to Notch signalling; the role of Notch-Delta signalling in the development and regeneration of HC and SC has been well-established (Chrysostomou et al., 2012; Daudet et al., 2009; Lanford et al., 1999). We thus treated electroporated BP explants with DAPT, an inhibitor of gamma-secretase, important in the activation of Notch. Treatment with DAPT did not affect HC restoration (Fig. 5I), and the numbers of HC in control or Atoh1-gRNA explants were higher than vehicle controls (Fig. 5J). Closer inspection revealed a greater number of HC-HC contacts in the DAPT-treated explants, irrespective of whether they were from control-gRNA or Atoh1-gRNA electroporation (Fig. 5K).

Signalling Activity during HC Restoration

To understand when and where signalling was active during HC restoration, we co-electroporated reporter constructs alongside Atoh1-gRNA CRISPR/Cas9 components. We first looked at Notch signalling. The pT2K-Hes5::nd2mScarlet plasmid consists of the mouse Hes5 promoter driving the expression of a destabilized and nuclear localized mScarlet fluorescent protein in Notch-On supporting cells (Fig. 6A)(Chrysostomou et al., 2012). mScarlet

expression is transient, with its presence limited to cells where Notch is active or has been active within the past 10 hours (Chrysostomou et al., 2012).

We observed a significant reduction in the population of Notch-On cells, expressing high levels of mScarlet, within the basilar papilla of Atoh1-gRNA crispants at E10 (Fig. 6B & 6D). However, a population of cells with lower mScarlet expression was observed at the luminal surface, where hair cells would normally be expected. Staining for phospho-Histone H3 (pH3), a marker of mitotic cells in cross sections of the basilar papilla, revealed a significant

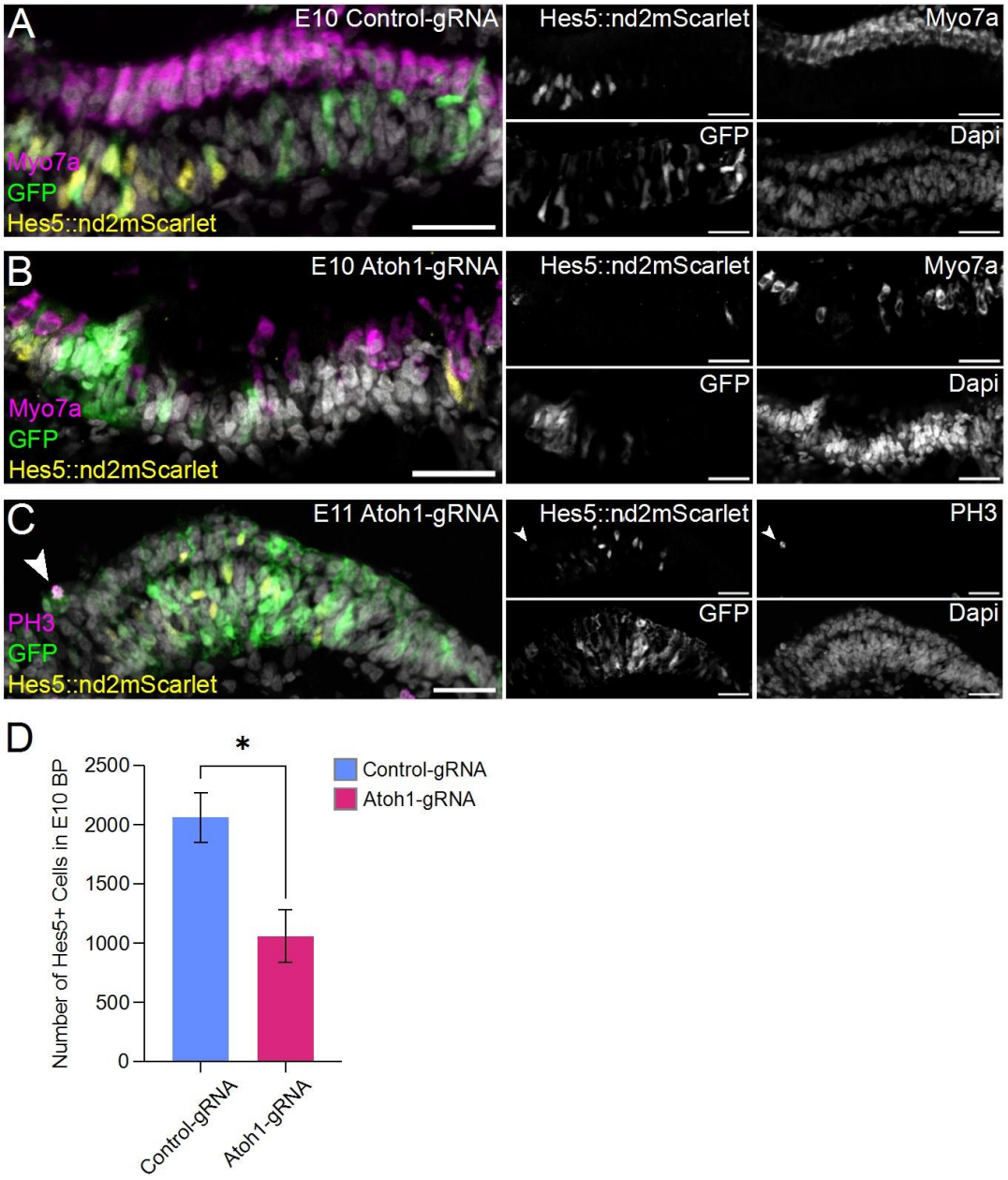

Figure 6: Hes5::nd2mScarlet reporter activity in Atoh1-crispant BP. Cross-sections of BP from electroporated chick embryos. (A, B) E10 BP showing Myo7a (magenta), Dapi-stained nuclei (grey) and Hes5::nd2mScarlett, Notch-on cells (yellow) and GFP (green) in control-gRNA electroporated BP (A) and Atoh1 electroporated BP. (Scale Bar is 25µm). (C) BP of Atoh1-gRNA at E11, which were co-electroporated with pT2K-Hes5::nd2mScarlet showing the mitotic marker Phospho-Histone H3 (PH3) (magenta), nuclei (grey), Notch-on cells (yellow), GFP (green). (Scale Bar is 25µm). (D) The graph shows the total number of Hes5+ cells in the E10 basilar papilla for the two conditions: control-gRNA and Atoh1-gRNA. P-values, with *P<0.05.

co-localization of pH3 positive cells with cells showing diminished mScarlet expression (Fig. 6C). As Hes5 activity is found in supporting cells, the reduced mScarlet expression in these cells may suggest a recent transformation into new hair cells. These findings indicate that the downregulation of Hes5 in supporting cells promotes hair cell differentiation and suggests that supporting cells can be induced to re-enter the cell cycle and give rise to new hair cells.

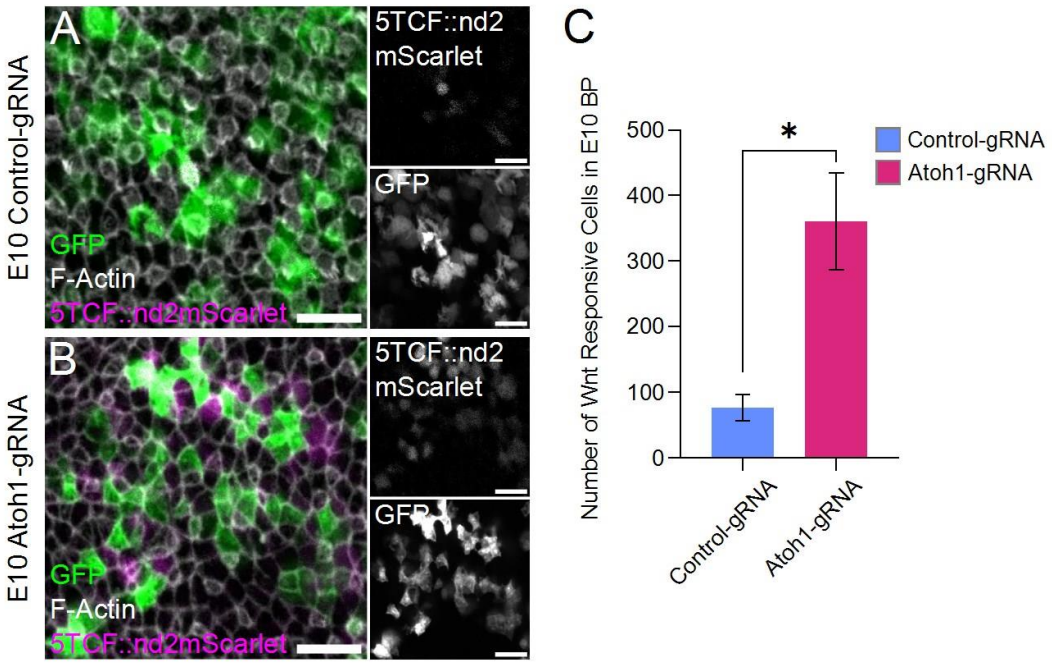

Figure 7: Wnt responsive cells in Atoh1-crispant BP. (A, B) Whole-mount E10 BP at E10 from electroporated chick embryos, showing 5TCF::nd2mScarlet Wnt-on cells (magenta), F-Actin (grey), and GFP (green) from control-gRNA BP (A) an Atoh1-gRNA electroporated BPs (B). (C) The graph showing the total number of Wnt-positive cells in the E10 basilar papilla for control-gRNA (blue) and Atoh1-gRNA (magenta). P-values - *P<0.05.

We next turned to the Wnt pathway, using co-electroporation of a Wnt reporter (pT2K-5TCF::nd2mScarlet). This construct consists of 5 TCF/LEF binding sites driving the expression of a destabilised, nuclear-localized mScarlet protein. Cells in which Wnt signalling

is active, or has been active in the past 10 hours, will show nuclear expression of mScarlet indicating Wnt receiving cells. We observed a significant increase in the number of Wnt-responsive cells within Atoh1-gRNA electroporated BP (Fig. 7B & 7C). These results are consistent with prior research in the field, which highlights the role of canonical Wnt signaling in promoting proliferation in lateral line regeneration (Jiang et al., 2014; Steiner et al., 2014).

Concluding Remarks

Hair cells of the inner ear of birds are replaced after damage. How the BP is able to sense the absence of a HC, what mechanisms are used to activate quiescent progenitors, and what are the steps in HC replacement remain unclear. This has been, in part, due to the inaccessibility of HC regeneration paradigms, requiring post-hatch stages of chicks. In this study, we identify a second wave of hair cell differentiation in the chick basilar papilla, that is revealed by mosaic deletion of Atoh1, and that may provide a model for HC regeneration. We show that even though HC numbers are depleted at E9 after Atoh1 CRISPR-mediate deletion, they are restored by E12. HC Restoration is achieved through non-transfected SC differentiating into HC. Anaylsis of the timing of restoration suggests that it occurs in a distal to proximal fashion mirroring the normal differentiation profile of BP hair cells. Newly generated HCs exhibit a smaller apical area at E12 stage. Some of these newly formed cells lack hair bundles but still express Myo7a. Additionally, we have observed disruptions in planar polarity in Atoh1-crispants, although polarity alignment is restored in the course of BP development (data not shown). The parallels between restoration and regeneration suggest that some aspects of HC regeneration can be modelled through restoration. Moreover, the tractability of the embryonic system to electroporation allows the introduction of constructs into the tissue as HC are restored.

Most hair cells have developed in the chick BP by E9.5 (Goodyear and Richardson, 1997). However, during normal development, around 1000 more hair cells are added over 3 days, such that E12 obtains the final HC number of around 12,000. Between E9 and E12, the BP is also undergoing cellular rearrangements, that are not only responsible for the extension of the BP, but also for the alignment of HC polarity (Goodyear and Richardson, 1997; Prakash et al., 2023). We therefore speculate that during normal development, a second wave of HC differentiation restores the correct number of HC neighbours to SCs that may have lost a neighbour during these rearrangements. At E12 SC have 2 HCs neighbours (Prakash et al.,

2023). In our Atoh1-crispants, there are a substantial number of SC that have no HC neighbour at E9, however by E12 most SC contact 2 HCs.

The mechanisms that sense an inconsistency in the numbers of neighbours is unclear. One obvious candidate would be the interaction between Notch1 (on supporting cells) with Dll1 (on hair cells). In this model, SC fate is maintained through the continued production of NICD after Notch interaction with its ligand (either Dll1, or Jag2) in the HC. In the absence of the ligand, SC differentiate into HC. Previous work suggests Notch signalling itself is not required for the release of quiescence during regeneration, and that DAPT treatment of the adult, undamaged, BP does not result in an increase in HC (Daudet et al., 2009). However it is likely that at embryonic stages, the some of the SC precursors are not quiescent. Consistent with this, we observe a down-regulation of the number of cells showing expression of mScarlet driven from the Hes5-ndmScarlet Notch reporter, in response to Atoh1 downregulation. Control explants treated with DAPT, a gamma-secretase inhibitor that inhibits Notch signaling, show a modest elevation of HC number compared to DMSO control. HC numbers are restored to this value in Atoh1-crispant BP explants. The elevation of HC number is modest, and restoration of HC number after Atoh1-gRNA electroporation is only to this elevated number. This modest elevation suggests that only a subset of SC may have progenitor activity.

In contrast to the suppression of Notch signalling during HC restoration, a canonical Wnt reporter is upregulated in response to mosaic Atoh1 deletion. However, how, and if, these two signalling pathways intersect is unclear. Inhibiting Wnt signalling, using XAV939 prevents the restoration of HC. This suggests that ß-catenin stabilization, as a result of Wnt signalling, is important for the maintenance of the progenitor fate for HC conversion in the BP, and in its absence these progenitor cells enter a quiescent state. In damaged neonatal mouse utricle, stabilized ß-catenin in Lgr5+ cells enhances mitotic activity and HC regeneration (Kuo et al., 2015). Arguing against this, is the effect of the Wnt agonist, CHIR99021. Rather than promoting quiescence, the CHIR compound leads to a modest elevation in HC numbers in both control-gRNA and Atoh1-gRNA electroporated samples compared to the vehicle-treated samples. CHIR99021 is an inhibitor of glycogen synthesis kinase 3 (GSK-3ß) which usually leads to a destablisation of ß-catenin. GSK-3ß can also phosphorylate NICD, protecting it from degradation (Foltz et al., 2002). Thus, it is possible that CHIR99021 could also lead to a degradation of NICD, in effect releasing the Notch-mediated maintenance of SC/progenitor fate. Additional work is required to determine these interactions, however combining mosaic

Atoh1 deletion with co-electroporation of signalling reporters and explant studies can allow imaging experiments and timed inhibitor studies to allow insights into the dynamic regulation of this process.

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